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(54) Title: METHOD FOR PREPARING, ISOLATING AND SEQUENCING POLYNUCLEOTIDES

(57) Abstract

The present invention provides a method for sequentially preparing, isolating and sequencing a polynucleotide acid strand comprising the steps of: (1) preparing a solution of a polynucleic acid target fragment having attached thereto a separating label, (2) mixing the solution with a support matrix having attached thereto a group cooperatively bindable with the separating label, (3) immobilising the polynucleic acid fragment on the support matrix via binding of the separating label and the group, (4) purifying the immobilised strand, and (5) treating the immobilised strand for sequencing, wherein the preparing step, the immobilising step and at least part of the treating step are conducted in the same vessel.

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# Method For Preparing, Isolating and Sequencing Polynucleotides

The present invention relates to a method of preparing, isolating and sequencing polynucleotides.

The method is particularly useful for isolating and sequencing polynucleotide fragments prepared by a modified polymerase chain reaction (PCR) technique that is disclosed in International patent application PCT/GB91/00803.

Since its introduction, the di-deoxy DNA sequencing method (Sanger *et al* [1977] PNAS 74 5463) has found wide spread acceptance and has even been successfully utilised for sequencing large viral genomes (Baer *et al* [1984] Nature 310 207). Numerous improvements have been made in an attempt to permit the elucidation of more than one DNA sequence from a single clone. Most of these improvements are related to improving the electrophoretic resolution and rapid computer assisted data entry. The advent of fluorescently labelled sequencing reagents has enabled the combination of both of these improvements, thereby permitting automation of electrophoresis and gel reading. However, because these improvements are aimed at facilitating gel running and data entry, the limiting factor in analysing polynucleotide fragments actually becomes the step of preparing the fragments for sequencing.

An improvement in target fragment preparation was reported by Kristensen (Kristensen *et al* [1987] NAR 15 5507). This improvement involves the precipitation of M13 phage with acetic acid, followed by the recovery and subsequent disruption of the phage on glass fibre discs. The released DNA is then eluted with a low salt buffer, which requires neither phenol extraction nor ethanol precipitation. This improvement is reported to have a potential for automation (Zimmerman *et al* [1989] Meth. Mol. Cell. Biol. 1 29) but suffers from the fact that it is limited to the use only of M13 phage and its derivatives.

Also, the improvement is further limited because it is dependent upon bacterial growth (which usually has to occur over a period of six hours) and a DNA purification step.

The polymerase chain reaction (PCR) (Saiki, R. *et al* [1985] Science 230 1350 and [1987] Science 239 487) offers one way of circumventing the need for having a bacterial culture for preparing amplified amounts of a target fragment for sequencing. However, there are some inherent problems associated with the PCR technique. For example, the products of the PCR are often relatively short linear molecules, which are usually less than 2kb in length. Also, the products cannot be directly sequenced because of the presence of residual dNTPs and primers in the reaction mixture. Also, there is a tendency for the separated DNA strands to reanneal very quickly after alkali or heat denaturation. However, reannealing can be hindered by using 10% DMSO (Winship [1989] NAR 17 1266).

Other methods to overcome the annealing problem include the complete removal of one strand or conducting asymmetric PCR to produce an excess of one strand (Gyllensten *et al* [1988] PNAS 85 7652). Because of the unexplained template and primer variability encountered in the Gyllensten method, an alternative method of single strand template preparation has been suggested (Higuchi *et al* [1989] NAR 17 5865).

The method of Higuchi depends on the phosphorylation of one of the PCR primers which then targets the strand to be digested, following PCR, by λ exonuclease III. Other workers have used $^{32}P$ end-labelled PCR or internal primers prior to the sequencing step (Wrischnik *et al* [1987] NAR 15 529, Wong *et al* [1987] Nature 330 384). Also, direct sequencing from low-melt agarose has been conducted (Kretz *et al* [1989] NAR 17 5864).

Accordingly, current techniques for the preparation, isolation and sequencing of nucleic acid fragments include a first step of amplifying a target nucleic acid fragment (i.e. the fragment of interest), for example, by use of the PCR technique (Saiki, R. *et al* [1985] Science 230 1350 and [1987] Science 239 487). Next, the copies of the target fragment produced by PCR amplification are isolated, for example, by gel electrophoresis. The isolated strands are then sequenced by use of standard di-deoxy labelling or radiolabelling techniques or standard chemical degradation techniques.

However, it is generally known and accepted that it is not practicable to conduct each of the present steps in a simple, reliable and straightforward run from start (i.e. preparing amplified amounts of the target fragment) to finish (i.e. sequencing the prepared target fragment). It is also accepted that it is not possible to have one machine that could carry out each of the above steps in a continuous run. This is because the amplification, isolation and sequencing steps are three quite separate and incompatible steps that have to be conducted in different vessels. Also, these steps have to be performed using quite different volumes of samples and concentration levels. Also, some of these methods require time consuming additional steps prior to sequencing and, often, the results from the sequencing analysis can vary according to the protocol used.

The major drawbacks that prevent such a reliable and continuous method from being devised therefore stem from the inherent problems associated with each of the present steps. For example, the amplification step often requires extremely dilute samples of the target fragments, whereas the isolation step thrives on concentrated samples. Also, each of the above steps using current methods employs solutions having volumes of approximately 0.01 ml and upwards, with the amplification step requiring the largest volumes.

Thus an automated machine using current techniques would have to handle large volumes. This is clearly disadvantageous because the machines themselves would be large and costly to produce, maintain and run.

It is therefore apparent that none of the forementioned methods is amenable to large-scale sequencing of genomic products such as those involved in Human Genome Project.

It would be extremely advantageous, therefore, to have a method of, and also a machine for, preparing, isolating and sequencing a target fragment. It would be even further advantageous to have such a method wherein each of those steps could be conducted at the μl level. In order to achieve this goal, the present method builds upon and refines a method devised by Hultman *et al* ([1989] NAR 17, 4937-4946).

The Hultman method was devised for the sequencing of polynucleotide fragments. The method yields a purified polynucleotide fragment attached to a solid support, which can then be readily sequenced by standard Sequenase (trade mark) or Taquenase (trade mark) methodology.

In brief, the Hultman method uses streptavidin coated magnetic beads to immobilise biotin labelled polynucleotide fragments. Once the fragments are immobilised, any unwanted products and artifacts can be easily removed by washing the beads. The fragments can thus be isolated and purified by a straightforward and simple procedure.

In brief, the Hultman method of immobilisation includes the following 6 steps:

1. preparing magnetic beads containing covalently coupled streptavidin (e.g. Dynabeads M280-Streptavidin, which can be obtained from Dynal AS, Norway),

2. preparing a target double stranded (ds) DNA fragment (e.g. from a plasmid or a sample of genomic DNA),

3. binding a biotin label to either the 5' end or the 3' end of one of the strands of the ds DNA fragment (see below),

4. mixing the prepared beads and the biotin labelled ds fragment under conditions to allow the fragment to bind to the beads via the biotin label (i.e. immobilising the labelled fragments),

5. isolating the complexed bead-fragments by use of a magnet (e.g. neodymium-iron-boron permanent magnet), and

6. optionally washing the isolated complexed bead-fragments.

The binding of the biotin label (step 3) can be performed by either a filling-in of nucleotide overhangs of a sticky-ended fragment with biotin-dUTP and polymerase (i.e. extension of the target strand) or by using a biotinylated primer that anneals to a universal cassette that is ligated to the target strand during an *in vivo* amplification operation (e.g. during an exponential PCR amplification).

The fragments prepared by the alternative biotin binding methods have different orientations when immobilised on the beads.

In this regard, the filling-in step using biotin-dUTP produces a biotin label at the 3' end of a strand of the target fragment. Therefore, when the target strand is immobilised on the beads, the 5' end of this target strand is distanced away from the beads.

In contrast, the use of a biotin labelled primer that anneals to a ligated universal cassette produces a biotin label at the 5' end of a template strand of the target fragment. Therefore, when this target strand is immobilised, the 3' end of the target strand is distanced away from the beads.

The fragments to which the biotin labels bind can be prepared, for example, by PCR exponential amplification. After PCR amplification, the biotin labelled strands of the fragments can be bound to the beads. The PCR products can be easily purified because the non-biotinylated strands of the fragments can be selectively removed by alkaline treatment. The purified polynucleotide fragment strand can then be sequenced.

In the Hultman method, the immobilised biotinylated polynucleic acid fragment strand is sequenced by a procedure including the following 12 steps:

1. denaturing the immobilised double stranded (ds) fragment to a single stranded (ss) polynucleic acid fragment with 0.15 M NaOH for 5 minutes,

2. washing the beads with 0.15 M NaOH and $H_2O$,

3. mixing the washed immobilised ss fragment with 2 pmol of a labelled (i.e. $^{32}P$ labelled or fluorescently labelled) sequencing primer in a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$ and 0.1 mg/ml BSA in a total volume of 0.01 ml,

4. heating the annealed mixture to 65°C and allowing it to cool to room temperature,

5. adding 0.001 ml DTT/NaCl (0.1 M DTT/0.8 M NaCl) and 4 units of T7 DNA polymerase, and then adjusting the volume to 0.015 ml,

6. mixing 0.0035 ml of the mixture from step 5 with 0.0025 ml of a respective nucleotide mixture (see below),

7. incubating the reaction mixture for 10 minutes at 37°C,

8. removing the supernatant after extension has been completed and washing the beads in water,

9. adding 0.003 ml of a formamide/sequencing dye mixture (deionised formamide consisting of 10 mM EDTA, pH 7.5 0.3% (w/v) xylen cyanol ff and 0.3% (w/v) Bromophenol Blue) and incubating at 37°C for 15 minutes,

10. removing the supernatant mixture (which includes the newly synthesised oligo-nucleotides) and diluting the mixture with 0.003 ml water,

11. loading 0.002 ml of the diluted supernatant mixture on a 4% standard electrophoresis polyacrylamide gel or on a 7 % sequencing gel, which is then run using an automatic sequencing apparatus, and

12. detecting the bands after electrophoresis.

The typical nucleotide concentrations and mixtures used in step 6 are 0.080 mM of each dNTP. 0.0063 mM of the respective ddNTP, 50 mM NaCl and 40 mM Tris-HCl (pH 7.5).

Despite the apparent simplicity of the Hultman method, there are a number of associated inherent problems with the immobilising and sequencing steps. In particular, the method requires large quantities of Taq polymerase, biotinylated primer and streptavidin beads. Also, the sequencing stage requires a labelled primer and a purified template.

We have considerably modified the Hultman method to overcome its inherent problems. In particular, our method is conducted on the micro volume scale thereby permitting a reduction in the amount of Taq enzyme, primer and streptavidin beads required.

Our method also allows the direct addition of λ phage, m13 phage or bacterial cells to the PCR reaction without any additional purification of the PCR template. Thus it is possible to circumvent the need for bacterial growth and DNA template preparation. This makes the whole process very amenable to automation.

Indeed, using the present method, it is possible to carry out a PCR amplification reaction or a number of the PCR amplification reactions in a specially designed microtitre plate PCR machine and to isolate the products therefrom and to sequence the products without having to transfer the products to different reaction vessels.

According to the present invention, we provide a method of sequentially preparing, isolating and sequencing a polynucleic acid strand comprising the steps of:

1. preparing a solution of a polynucleic acid target fragment having attached thereto a separating label,

2. mixing the solution with a support matrix having attached thereto a group cooperatively bindable with the separating label,

3. immobilising the polynucleic acid fragment on the support matrix via binding of the separating label and the group,

4. purifying the immobilised strand, and

5. treating the immobilised strand for sequencing,

wherein the preparing step, the immobilising step and the (or at least part of the) treating step are conducted in the same vessel.

Preferably, all of the sequencing reaction is conducted in the same vessel and the product is then sequenced on an automated machine, such as Du Pont's Genesis 2000 machine. This can be achieved by use of fluorescent dideoxy chain terminators. The products of the treating step need only then be run on one gel in order to establish the sequential order of the nucleotides in the immobilised strand.

It is appreciated that if non-labelled dideoxy terminators are used in the treating step in conjunction with $\alpha^{35}S$ labelled dideoxy terminators, the immobilised strands could then be subjected to the respective radiolabelled terminators in different vessels and at different times.

Preferably, the polynucleic acid target fragment is prepared by a PCR amplification step, Advantageously, the PCR amplification is conducted in a volume of between 5$\mu$l and 20$\mu$l, preferably 10$\mu$l.

Advantageously, one of the primers used in the amplification step has attached thereto the separating label. Preferably, the separating label is attached to the 5' end of the primer. Conveniently, the primer with the attached label is a primer that anneals to a specific region on the target fragment. This ensures that only the target fragment is

isolated.

Preferably, the label is biotin and the support matrix is a magnetic bead having streptavidin covalently coupled thereto. The streptavidin beads are particularly advantageously because harsh conditions (e.g. strong alkaline solutions) can be used to clean the immobilised fragments without the immobilised fragments becoming unbound from the beads.

However, it is to be understood that any other appropriate separating label and support matrix would suffice, for example DNA binding proteins.

Preferably, the beads are added in the form of an aqueous dispersion to the amplification mixture. A typical aqueous dispersion has a volume of 15$\mu$l.

Advantageously, if the labelled target fragment is a double stranded fragment, it can be denatured while it is bound to the support matrix. However, it is clear that the target fragment can be denatured prior to the addition of the support matrix.

In both instances, only the labelled target fragments will bind to the support matrix. A typical denaturation solution is 20$\mu$l of 0.15M NaOH. The fragments bound to the beads can then be washed with, approximately, 20$\mu$l solutions of 0.15M NaOH and 40$\mu$l solutions of $H_2O$.

Advantageously, the purified fragments attached to the beads are dispersed in approximately 5-10$\mu$l $H_2O$ ( preferably 7.5$\mu$l) and sequenced using approximately 5-12 $\mu$l of sequencing solution (preferably 7-10$\mu$l). A typical sequencing solution comprises approximately 0.5$\mu$l of 10$\mu$M forward primer, 2$\mu$l of 5x Sequenase buffer, 1$\mu$l of 100mM DTT, 2$\mu$l of labelling mix (radiolabelled dideoxy terminators or fluorescent dideoxy terminators), 0.5$\mu$l of $^{35}S$ dATP and

2μl of Sequenase; 4μl of which is mixed with 2.5μl of termination labelling mix.

The present method is suitable for a simple kit so that the method can be conducted on a laboratory workbench. The present method can also be conducted by an automated machine, particularly as the amounts of reagents required are low. Also, such an automated machine could be supplied in kit form.

The present method has a considerable number of advantages over existing methods. It is quicker than conventional methods, it yields better quality sequences using either $^{35}S$ and autoradiography or fluorescent di-deoxy chain terminators (e.g. with the Du Pont Genesis 2000™ sequencing machine) and it allows the rapid sequencing of any nucleic acid fragment, for example genomic DNA that can be, but need not be, cloned in a vector.

Also, the present method makes it possible to prepare the template DNA, perform the sequencing reactions and run a sequencing gel in one working day. In addition, the data obtained using the present method are of higher quality than those obtained from either conventional double or single stranded DNA sequencing.

Moreover, the isolated target fragment template produced is extremely clean and therefore the sequence produced is also very clean. This is true both of $^{35}S$-labelled DNA and fluorescently labelled fragments.

A method according to the present invention will now be described by way of example only.

Step 1. Preparation by PCR amplification

The target fragment to be sequenced (which may be contained within λ phage, M13 phage, yeast or bacterial cells) is added to the 10μl reaction mixture, which comprises 1μl of 10x Cetus buffer, 0.5μl of 10μM biotinylated reverse primer, 1μl of 2mM dNTPs and 0.04 units of Taq polymerase. $H_2O$ is then added so that the total volume is 10μl.

Next the mixture is overlayed with 10-20μl light paraffin oil, then heated at 95°C for 0.3 min, 55°C for 0.5 min and 72°C for 1 min for a total of 35 cycles, followed by heating at 72°C for 3 min.

Step 2. Isolation of labelled target fragment

15μl of streptavidin coated magnetic beads in TE/NaCl are added to the whole of the PCR reaction (including paraffin). After mixing, the mixture is incubated at room temperature for 15 min.

Then, optionally, the beads are washed four times with TE/NaCl.

Step 3. Denaturation

The beads are incubated at room temperature for 5 min with 20μl of 0.15M NaOH. The beads are then washed with 20μl of 0.15M NaOH (if desired, the paraffin can be wholly or partially removed at this stage) and then three times with 40μl of $H_2O$.

Step 4. Sequencing

The beads are resuspended in a total volume of 7.5μl of $H_2O$, to which suspension is added 0.5μl of 10μM forward primer and 2μl of 5x Sequenase buffer. The mixture is incubated at 65°C for 2 min, then ice cooled for 1 min. Next, 1μl of

100mM DTT, 2μl of diluted labelling mix (1:5 for longer sequences, 1:20 for shorter ones), 0.5μl of $^{35}$SdATP (0.5μCi) and 2μl of diluted Sequenase (1:8) are added.

The mixture is then incubated at room temperature for 5 min. 4μl is transferred to the termination mixes (2.5μl). The mixture is incubated at 37°C for 5 min and then washed with 20μl TE. Afterwards, the supernatant is removed and 4μl of formamide dye mix is added. Following incubation at 80°C for 5 min, the mixture is spun and the supernatant removed. A sample (e.g. 3-4μl) of the supernatant is then loaded on a buffer gradient sequencing gel.

The present method can be easily conducted in the laboratory. In a typical example, a 96-prong replica-plated comb transfers bacterial cells into the wells of a thermostable polycarbonate microtiter plate. A programmed robotic workstation dispenses 10μl of PCR mixture (200μM dNTP's, 1.5mM $MgCl_2$, 50mM Tris-HCl pH 8.3, 0.01% gelatin, 1μM each of M13 Universal forward and reverse sequencing primers, 0.8 Units Taq polymerase USB™) into the microtiter wells and, following plating, overlays the solution with 40μl light mineral oil.

Next, the plate is incubated on a modified programmable thermocycler adapted to accommodate such plates. The target DNA is then exposed by heating the solution to 95°C for 2 minutes prior to 30 cycles of amplification (95°C - 0.5 min, 55°C - 0.5 min, 72°C - 1 min).

This present method allows the presence and size of inserts in 96 clones to be determined within 4 hours.

We have also extended this protocol to the direct sequencing of PCR products in which the amplification involves one biotinylated primer. This product is bound to magnetic streptavidin beads and washed with NaOH and $H_2O$. The single-stranded fragment templates can be routinely

sequenced either manually, using Sequenase™, or in a single well with fluorescently labelled dideoxynucleotides, prior to loading on a Du Pont Genesis 2000 automated DNA sequencing machine.

It is clear from the above, that the present method includes the small-scale amplification of DNA template in which one of the PCR primers has a biotin group at its 5' prime end. This allows a single strand of DNA to be purified by binding it to streptavidin-coated magnetic beads and washing with alcohol. Because the reactions are carried out on a small scale, the amount of Taq polymerase enzyme required is kept to a minimum, thus reducing cost.

It is clear that the rapid automated screening and sequencing strategies described here will contribute to both small and large scale sequencing projects.

It will of course be understood that the present invention has been described above by way of example only and that modifications and variations can be made by the skilled person without departing from the scope of the invention.

CLAIMS

1. A method for sequentially preparing, isolating and sequencing a polynucleotide acid strand comprising the steps of:

(1) preparing a solution of a polynucleic acid target fragment having attached thereto a separating label,

(2) mixing the solution with a support matrix having attached thereto a group cooperatively bindable with the separating label,

(3) immobilising the polynucleic acid fragment on the support matrix via binding of the separating label and the group,

(4) purifying the immobilised strand, and

(5) treating the immobilised strand for sequencing,

wherein the preparing step, the immobilising step and at least part of the treating step are conducted in the same vessel.

2. A method according to claim 1, wherein the entire treating step is conducted in a single vessel and sequencing is performed using an automated sequencing machine.

3. A method according to claim 1 or claim 2, wherein the polynucleic acid target fragment is prepared by a PCR amplification step.

4. A method according to claim 3, wherein the target fragment is prepared by performing a PCR amplification directly on M13, λ phage or bacterial cells.

5. A method according to claim 3 or claim 4, wherein the separating label is comprised in a primer used in the PCR amplification step.

6. A method according to any preceding claim wherein the separating label is biotin and the support matrix comprises a magnetic bead covalently coupled to streptavidin or avidin.

7. A method according to any preceding claim wherein step 4 comprises denaturing the target fragment in an alkali solution to purify the labelled strand.

8. A method according to any preceding claim wherein sequencing is carried out in a volume of less than 22μl.

9. A method according to any preceding claim which is conducted on a microlitre volume scale.

10. A method according to any preceding claim which is conducted substantially entirely by an automated machine.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01379

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68

## II. FIELDS SEARCHED

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
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| Int.Cl. 5 | C12Q |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No.[13] |
|---|---|---|
| X | NUCLEIC ACIDS RESEARCH. vol. 17, no. 22, 25 November 1989, ARLINGTON, VIRGINIA US page 9498; J.P. SCHOFIELD ET AL.: 'A rapid semi-automated microtiter plate method for analysis and sequencing by PCR from bacterial stocks' see the whole document | 1-10 |
| A | EP,A,0 371 437 (ORION-YHTYMA OY) 6 June 1990 see column 5, line 15 - column 15, line 25 | 1-10 |

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## IV. CERTIFICATION

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category ° | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| A | NUCLEIC ACIDS RESEARCH.<br>vol. 17, no. 13, 1989, ARLINGTON, VIRGINIA US<br>pages 4938 - 4946;<br>T. HULTMAN ET AL.: 'Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support'<br>cited in the application<br>see the whole document | 1 |
| A | BIOTECHNIQUES<br>vol. 9, no. 1, July 1990, EATON PUBL. CO.,NATICK,MA,USA<br>pages 32 - 36;<br>B.F. KOOP ET AL.: 'Sequencing reactions in microtiter plates'<br>see the whole document | 1 |
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| T | DNA SEQUENCE<br>vol. 1, no. 4, 1991, HARWOOD ACADEMIC PUBLISHERS, UK<br>pages 279 - 283;<br>D.S.C. JONES ET AL.: 'Fluorescent and radioactive solid phase dideoxy sequencing of pcr products in microtiter plates'<br>see the whole document | |

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9101379 SA 50445

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 13/11/91
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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP-A-0371437 | 06-06-90 | AU-A- 4574989 | 07-06-90 |
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| | | JP-A- 2219600 | 03-09-90 |